REMARKS

In the Office Action dated February 17, 2006, the Examiner rejected claims 1, 3, 5-8, 10 and 12-14 under 35 U.S.C. §103(a) as being unpatentable over Heidt, Copeland, Kerr, or Rogers in view of Potter.

In the most recent Amendment filed on August 17, 2006, Applicants noted that none of the references cited by the Examiner, alone, or in combination, teach, disclose, or suggest, the claimed invention. Furthermore, none of the references provide a motivation to combine Potter with Heidt, Copeland, Kerr, or Rogers. Additionally, as discussed with Examiner Alexander in the interview on October 5, 2006, the Potter reference cited by the Examiner is nonanalogous art.

Potter is nonanalogous art

MPEP 2141 01(a), quoting *In re Oetiker*, 977 F.2d 1443, 1446 (Fed. Cir. 1992), states that "[i]n order to rely on a reference as a basis for rejection of an applicant's invention, the reference must either be in the field of applicant's endeavor or, if not, then be reasonably pertinent to the particular problem with which the inventor was concerned."

Potter is not in the field of Applicants' endeavor

Both Potter and the present invention are pieces of laboratory equipment. However, that is where the similarity ends. In the context of the kinds of laboratories in which the two are used, the functions for which they are used, the kinds of samples on which they act, the functional requirements each need in processing the samples, and the staff who would use them, the two are far apart.

Potter relates to the field of laboratory analysis of liquid samples, such as DNA or RNA amplification, carrying out enzyme reactions, and investigating rates of hybridization and melting of nucleic acids. (See column 1, line 9 through column 2, line 15). The Potter device is commonly known in the art as a "PCR cycler," and is typically used for performing polymerase chain reaction. Potter discloses a PCR cycler in which different reactions in adjacent vessels can

apparently cycle temperature independently of one another. The Potter device would most commonly be found in a molecular biology laboratory. Devices like this are commonly used for amplifying DNA, whereby DNA strands are copied using DNA polymerase, "melted" (separated) by heating, and then copied again, repeatedly. This process of repetitively copying DNA by cycling the temperature can produce logarithmic amplification of the starting DNA template. The amplified DNA can then be used for another purpose such as for insertion into a plasmid (as in genetic engineering). Alternatively, it can be labeled with a reporter molecule and used as a molecular probe or target. The amplified DNA can be analyzed for its presence, such as in a binding assay, or for its sequence (by DNA sequencing). Such reactions are conventionally performed in solution, not on microscope slides with tissue sections or cells. Consequently, the Potter device requires vessels for containing liquid, and is not compatible with conventional flat microscope slides. The type of staff who would work with such a device would typically be trained in working with nucleic acids, such as genetic engineering or molecular biology.

The present invention, on the other hand, relates to the field of slide processing. The claimed apparatus is designed for use with microscope slides bearing a biological sample. Similarly, the methods claimed in the present invention involve microscope slides bearing a biological sample. Slide stainers such as described in the present invention are most commonly found in histology laboratories. They are used for coloring tissue sections or cells so that morphologic details of the tissue or cellular structure can be observed under the microscope. Slide stainers must, of necessity, work with flat slides since the biological sample mounted on the slide must be kept in a single plane for optical inspection under the microscope. The type of staff who typically work with such a device have a background in histotechnology and can be certified by the American Society of Clinical Pathologists in that field. Such individuals often write "HT" (histotechnology) after their names when used in a professional context, to designate their professional certification. This field involves the processing of tissues, such as biopsies, so that they can be cut into extremely thin slices and then stained. Most commonly, this is done on patient samples for diagnostic purposes.

Potter is not reasonably pertinent to the particular problem concerning Applicants'

As described previously, Potter discloses an apparatus capable of independently regulating the heating of all samples in a sample container designed for rapid heat transfer to a set temperature. The apparatus is used to facilitate chemical reactions within liquid samples. Furthermore, the liquid samples, once placed in the reagent wells, are sealed off from the outside environment. Potter does not disclose an apparatus for use with microscope slides, having a liquid dispenser, or that causes relative motion between a liquid dispenser and a platform.

At the time of the invention, Applicants' were concerned with enabling random access processing of multiple microscope slides in different ways by applying the reagent to selected slides. An apparatus incapable of random access processing or applying reagents to the slides is not pertinent to this concern.

Thus, because Potter does not relate to the field of slide processing and because Potter is not reasonably pertinent to the particular problems of random access slide staining that concerned the Applicants, Potter is not analogous art. The rejection under 35 U.S.C. §103(a) is therefore traversed, and reconsideration is requested.

There is no motivation to combine

MPEP 2403.01 provides that "[o]bviousness can only be established by combining or modifying the teachings of the prior art to produce the claimed invention where there is some teaching, suggestion, or motivation to do so found either explicitly or implicitly in the references themselves or in the knowledge generally available to one of ordinary skill in the art."

In the Office Action dated February 17, 2006, the Examiner stated that "Potter et al teach in the abstract and columns 1-2 that it is desirable to heat slides independently based upon the specific reactions/conditions required for each slide" and that it would have been obvious to modify Heidt, Copeland, Kerr, or Rogers in view of Potter "and use individual heating elements and control to gain the advantages of tailoring the precise temperature for the application of interest."

Potter does not provide motivation for combination with the primary references

It is respectfully submitted that the Examiner has mischaracterized Potter. Potter does not mention slides, nor does it relate to the field of slide staining. Instead, Potter, as discussed previously, describes an apparatus to be used with liquid samples. DNA or RNA amplification (also known as polymerase chain reaction, or "PCR") is not an accepted procedure for tissue sections or cellular samples mounted on microscope slides. As the nucleic acid is amplified, the newly-formed nucleic acid copy, also termed "amplicon," diffuses away from the cell in which it was formed, defeating the purpose of performing it on a tissue section. Similarly, enzyme kinetics and melting curves of nucleic acids are conventionally studied in solution, not in tissue. Immobilizing enzymes or nucleic acids, such as when they are in tissue sections, unpredictably changes those parameters as compared to when performed in solution. When enzymes are in tissue, the rate of the reaction (kinetics) is not only due to the inherent reaction rate of the enzyme but is also associated with the ability of the enzyme substrate to get into the tissue. Also, other tissue elements may interfere with the enzyme, slowing it down for reasons having nothing to do with the enzyme's inherent reaction rate. The same analysis also applies to hybridization and melting of nucleic acids. Consequently, it would be unusual for one of skill in the art to study enzyme reactions or nucleic acid hybridization or melting when the enzyme or nucleic acid is in tissue. For these reasons, the heating control taught by Potter is not meaningful in the context of slide staining. The benefits of plural heating surfaces in a PCR cycler as proposed by Potter do not translate to meaningful benefits in the field of slide staining.

Additionally, because Potter does not relate to slide staining or analyzing chemical analyte slides, one of ordinary skill in the art would not have been motivated to combine any of the devices disclosed in any of the primary references with Potter.

The primary references do not provide motivation for combination with Potter

The primary references suggest no "advantages of tailoring the precise temperature control" suggested by the Examiner. In fact, such temperature control would be contrary to the teachings of the references.

Heidt and Kerr both disclose devices for use with chemical analyte "slides" used in clinical chemistry for measuring the concentrations of various chemicals in blood. Analysis of these chemical analyte slides does not require the heating control as disclosed in Potter. In fact, in order to accurately analyze such slides, the temperature at which each slide is incubated must be constant. The heating control of Potter would be contrary to the reaction conditions required for the primary references.

Rogers and Copeland disclosed a slide staining apparatus in which the slides are heated to the same temperature. In order to include the heating control of Potter, one of ordinary skill in the art would have had to significantly modify Rogers or Copeland. At the time of the invention, one of skill in the art would not have undertaken such a difficult and costly modification because there was no recognized benefit for plural heated surface areas, each heated by an electric heater thereunder in a random access dispensing system.

The state of the art of slide staining, at the time of the invention, did not provide motivation to look to the heating control of Potter

Routine Staining

Routine staining is preformed as a batch process where all slides are treated the same. The slides are typically mounted in baskets that are dipped into buckets of solution. As such, they do not require random access dispensing systems as claimed. Further, they generally do not require heating. Thus, routine staining would not benefit from the slide handling assemblies of the primary references or from the heating of Potter.

Advanced Staining

There are three general categories of advanced staining, commonly known as special stains, immunohistochemistry, and *in situ* hybridization. At the time of the invention, one of ordinary skill would not have predicted the utility of plural heated surface areas, each heated by an electric heater thereunder, in a random access dispensing system for any category of slide staining.

Special Stains

At the time of the invention, special stain techniques often required judgments on the part of the technician, such as color analysis. Namely, the technician dipped the slide in a chemical or dye until the tissue elements acquired a certain specified color, as determined visually. Examples of special stain processes are presented in Exhibits A, B and C. In those exhibits, arrows with asterisks indicate steps in the procedures which must be performed visually and thus require user input. Because such techniques rely highly on the skills of the technician, and are considered an art, they had not been considered appropriate for automatic processing using a carousel type system.

Immunohistochemistry

The second type of advanced stain is immunohistochemistry. As practiced in 1994, immunohistochemical slides were either processed at room temperature (without the application of heat) or were heated to approximately body temperature. In either situation, all of the slides were processed at the same temperature, regardless of the particular histochemical stain. The automated slide stainers on the market by 1994 did one or the other. Examples of automated slide stainers without any heating capability were Fisher's Code-on and Shandon's Cadenza; whereas, Ventana's 320/ES immunohistochemical slide stainer (similar to the Copeland primary reference) heated all of the slides to approximately body temperature. The inclusion of plural heated surface areas, each heated by an electric heater thereunder in a random access dispensing system imposes significantly greater technical challenges and expense. As such, one of ordinary skill in the art would need a compelling reason to undertake the technological and economic challenges associated with such modifications. In 1994 (the year of the invention), no such compelling need existed.

in situ Hybridization

The third category of advanced staining is *in situ* hybridization (ISH). This type of stain requires temperatures that are much higher than body temperature, often in the 70-95°C range. However, the small volume of reagent probe typically used (approximately ten microliters) can rapidly evaporate at such temperatures. To prevent evaporation during ISH processing, the tissue

section and the small amount of reagent/probe are sealed on the slide using a coverslip. The edges of the coverslip are sealed with, for example, rubber cement or nail polish. As discussed previously, a system in which the sample must be sealed off from the outside environment is mechanically incompatible with devices that control relative movement between a dispenser and slide support and that dispense reagent by dropping it onto a slide, such as in the claimed stainer. One of ordinary skill at the time of the invention (1994) would not have considered the high temperature heating, stringent requirement for preventing evaporation of an extremely low volume of reagent, and open dispensing system as compatible with each other.

Thus, one of skill in the art would not have been motivated to adapt the heating control described in Potter to the devices described in Heidt, Copeland, Kerr, or Rogers. Selectively heating slides, while enabling random access processing, involves technical challenges not addressed by Potter. Because there is no motivation to combine the references, the rejection under 35 U.S.C. §103(a) is respectfully traversed and reconsideration is requested.

CONCLUSION

In view of the prior amendments and remarks and the above remarks, it is believed that all claims are in condition for allowance, and it is respectfully requested that the application be passed to issue. If the Examiner feels that a telephone conference would expedite prosecution of this case, the Examiner is invited to call the undersigned.

Respectfully submitted,

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Manual of HISTOLOGIC STAINING METHODS of the Armed Forces Institute of Pathology

Third Edition

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18. Rinse thoroughly in three changes of absolute alcohol. It is essential not to use low grade alcohols. If low grade alcohols are used, the cytoplasmic stains are dissolved and the tissue will not take on the collagen stain, which is made up in absolute alcohol.

19. Alcoholic safran solution for 5-15 minutes.

20. Dehydrate in three changes of absolute alcohol, and clear in several changes of xylene. If the collagen is not sufficiently yellow, repeat the staining with safran.

21. Mount with Permount or Histoclad.

RESULTS

Nuclei	- black
Elastic fibers	- dark purple to black
Collagen and reticulum fibers	- yellow
Ground substance	- blue to bluish green
Fibrinoid	- intense red
Muscle	- red

REFERENCE. Movat, H. Z.: *Arch. Path.*, 60:289-295, 1955.

JONES' METHOD FOR KIDNEY

FIXATION. 10% buffered neutral formalin, Bouin's or Zenker's

TECHNIQUE. Cut paraffin sections at 2 microns

SOLUTIONS

0.5% PERIODIC ACID SOLUTION

(See page 72)

3% METHENAMINE* SOLUTION

Hexamethylenetetramine (methenamine)	3.0 gm
Distilled water	100.0 ml

5% SILVER NITRATE SOLUTION

(See page 91)

BORATE BUFFER SOLUTIONS (STOCK)

Solution A: 0.2 M Boric Acid

Boric acid	12.36 gm
Distilled water	1000.0 ml

Solution B: 0.25 M Sodium Borate

Sodium borate	19.07 gm
Distilled water	1000.0 ml

*Fisher Scientific Co. or Eastman Kodak Co.

BORATE BUFFER SOLUTION, pH 8.2 (WORKING)

Solution A ..... 6.5 ml
Solution B ..... 3.5 ml

1% GOLD CHLORIDE SOLUTION (STOCK)

(See page 90)

GOLD CHLORIDE SOLUTION (WORKING)

Gold chloride stock solution ..... 10.0 ml
Distilled water ..... 40.0 ml

Solution is stable for approximately 100 slides.

3% SODIUM THIOSULFATE (HYPO) SOLUTION

Sodium thiosulfate ..... 3.0 gm
Distilled water ..... 100.0 ml

METHENAMINE SILVER SOLUTION, pH 8.2 (WORKING)

Methenamine, 3% ..... 42.5 ml
Silver nitrate, 5% ..... 2.5 ml
Borate buffer, pH 8.2 ..... 12.0 ml

Prepare fresh just before use and filter. This solution is stable for approximately 60-75 minutes. After this time, there is a breaking down process, which produces a black precipitate and is picked up on the slides

STAINING PROCEDURE. Chemically clean glassware must be used.

Note. It is absolutely essential that all glassware be acid cleaned with concentrated nitric acid and rinsed in several changes of chloride free distilled water. Distilled water may be checked for free chloride by the addition of several drops of 5% silver nitrate solution. If a white cloud appears upon the addition of the silver nitrate, discard the sample of water and replace.

1. Deparaffinize and hydrate to distilled water.
2. Periodic acid solution for 11 minutes.
3. Rinse in chloride free distilled water.
4. Filter freshly prepared methenamine-silver solution into coplin jar
5. Place slides in methenamine-silver solution and then place coplin jar in pre-warmed 70 °C water bath. Start timing at this point, approximately 60-75 minutes Check under microscope when slides show macroscopically a medium brown color.

Note. Solution and slides should be allowed to come to 70 °C together. While slides are in the silver solution they may be examined after they begin to show macroscopically a medium brown color reaction. Before checking under the microscope, they are first rinsed in hot 70 °C chloride free distilled water, checked, and then returned to hot water rinse and then returned into hot staining solution. Slides should be checked every 10 minutes when they have reached the dark or medium brown stage Slides should be checked as rapidly as possible because if the section cools there is an un-

even staining of the section. When the desired staining time has been reached, the slide should be checked as described above, every 1-2 minutes. Strict adherence to the timing is essential in order to obtain a uniform consistency in staining. A properly stained section at this point should have a dark brownish-yellow background; the reticulum fibers will be intense black, as should the basement membranes. An overstained section will be too black. Differentiation will be very difficult as the black will be so intense as to obscure many or all of the tissue elements. The section may be destained with an extremely dilute solution of potassium ferricyanide for one or two dips.

6. Rinse section well in distilled water.

7. Tone in working gold chloride solution for 1 minute.

Note. If sections are overtoned, place in 3% sodium metabisulfite for 1-3 minutes, checking periodically.

8. Rinse well in distilled water.

9. Sodium thiosulfate solution for 1-2 minutes

10. Wash in running tap water for 10 minutes

11. Rinse well in distilled water

12. Counterstain with routine Harris hematoxylin and eosin stain.

13. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, three changes each

14. Mount with Permount or Histoclad.

RESULTS

Basement membrane	- black
Reticulum fibers	- black
Nuclei	- blue
Cytoplasm, collagen, and connective tissue	- pink to orange

REFERENCE. Jones, D. B.: *Amer. J. Path.* 27:991-1009, 1951. Modified by Avalone, F., C. U. Branch, Armed Forces Institute of Pathology.

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Laboratory Methods in Histotechnology

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VERHOEFF'S ELASTIC STAIN

FIXATION: 10% buffered neutral formalin or any other well-fixed tissue.

SECTIONS: Paraffin, 6 micrometers.

SOLUTIONS

10% ALCOHOLIC HEMATOXYLIN SOLUTION (Ch. 3)

10% FERRIC CHLORIDE SOLUTION (Ch. 3)

VERHOEFF'S IODINE SOLUTION

Iodine ..... 2.0 gm
Potassium iodide ..... 4.0 gm
Distilled water ..... 100.0 ml

Mix the crystals of iodine and the crystals of iodide in a flask. Shake vigorously. Then gradually add the distilled water, 20 ml at a time.

VERHOEFF'S ELASTIC STAIN WORKING SOLUTION

Alcoholic hematoxylin, 10% ..... 25.0 ml
Alcohol, 100% ethyl ..... 25.0 ml
Ferric chloride, 10% ..... 25.0 ml

Mix well, then add:

Verhoeff's iodine solution ..... 25.0 ml

2% FERRIC CHLORIDE DIFFERENTIATING SOLUTION

Ferric chloride, 10% ..... 20.0 ml
Distilled water ..... 80.0 ml

VAN GIESON SOLUTION (Ch. 3)

5% SODIUM THIOSULFATE (HYPO) SOLUTION (Ch. 3)

PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Stain in Verhoeff's elastic stain working solution for 15 minutes.
3. Wash in lukewarm running tap water for 20 minutes.
4. Place in distilled water.
5. Differentiate in 2% ferric chloride solution. Check microscopically.[a] Elastic fibers are black and sharply fined; the background is gray.
6. Place in 5% sodium thiosulfate solution for 1 minute.
7. Wash in tap water for 5 minutes.
8. Place in distilled water.
9. Counterstain in van Gieson solution for 1 minute.[b]

10. Dehydrate rapidly[c] through 95% ethyl alcohol (2 changes) and absolute ethyl alcohol (2 changes); clear in 2 changes of xylene.
11. Mount with resinous medium.

RESULTS

Elastic fibers	black
Nuclei	black
Collagen	red
Other tissue structures	yellow

[a]Wipe the back of the slide. While wet with the 2% ferric chloride differentiation solution, check under low power. Elastic fibers in arterial walls should be black and the arterial wall muscle, gray.

[b]Do not leave in van Gieson solution for more than 1 minute. The picric acid component decolorizes the elastic fibers.

[c]Rinse rapidly in 95% ethyl alcohol to avoid decolorizing the van Gieson solution.

REFERENCE

Mallory FB. *Pathological Technique*. Philadelphia, PA: WB Saunders, 1942:170-171.

LEVADITI-MANOVELIAN METHOD FOR SPIROCHETES

FIXATION. 10% buffered neutral formalin. Specimen should be 1 mm thick

TECHNIQUE. Embed in paraffin after staining is completed (see Staining Procedure Step 10).

SOLUTIONS

3% SILVER NITRATE SOLUTION

Silver nitrate	3.0 gm
Distilled water	100.0 ml

REDUCING SOLUTION

Pyrogallic acid	4.0 gm
Formalin, 37 – 40%	5.0 ml
Distilled water	100.0 ml

STAINING PROCEDURE

1. Rinse specimen in tap water, after fixation.
2. Let stand in 95% alcohol for 24 hours
3. Transfer to distilled water and leave until the tissue sinks to the bottom of the container.
4. Place in freshly prepared silver nitrate solution and keep in 37°C in the dark for 3 to 5 days, changing the solution three times.
5. Rinse in distilled water.
6. Reducing solution at room temperature, in the dark for 24 to 72 hours.
7. Rinse in distilled water.
8. Dehydrate in 80% alcohol, 95% alcohol, and absolute alcohol, two changes, 30 minutes each.
9. Clear in oil of cedarwood for two changes, 1 hour each and infiltrate with two changes of paraffin 45 minutes each
10. Embed in paraffin.
11. Cut sections at 5 microns and mount on slides.
12. When dry, deparaffinize with xylene, three changes
13. Mount with Permount or Histoclad.

RESULTS

Spirochetes - black
Background - yellow to light brown

REFERENCE Mallory, F. B., *Pathological Technique*, New York, Hafner Publishing Co., 1961, p. 293.

WARTHIN-STARRY METHOD FOR SPIROCHETES AND DONOVAN BODIES

FIXATION. 10% buffered neutral formalin. *Avoid chromate fixatives*

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS. Use chemically clean glassware.

ACIDULATED WATER

Triple distilled water ..... 1000.0 ml

Add enough 1% aqueous citric acid to bring water to pH 4.0.

1% SILVER NITRATE SOLUTION (For impregnation)

Silver nitrate, C.P. crystals ..... 1.0 gm
Acidulated water ..... 100.0 ml

2% SILVER NITRATE SOLUTION (For developer)

Silver nitrate, C.P. crystals ..... 2.0 gm
Acidulated water ..... 100.0 ml

5% GELATIN SOLUTION

Sheet gelatin, high grade ..... 10.0 gm
Acidulated water ..... 200.0 ml

0.15% HYDROQUINONE SOLUTION

Hydroquinone, crystals, photographic quality ..... 0.15 gm
Acidulated water ..... 100.0 ml

Keep 2% silver nitrate, 5% gelatin, and 0.15% hydroquinone in 50 ml Erlenmeyer flasks, in a flotation bath at 54 °C until developer is made

DEVELOPER SOLUTION

Silver nitrate solution, 2% ..... 1.5 ml
Gelatin solution, 5% ..... 3.75 ml
Hydroquinone solution, 0.15% ..... 2.0 ml

Combine in the order given in small beaker, making certain solutions are mixed well. *Prepare immediately before use*

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to triple distilled water.
2. Impregnate with silver nitrate solution heated in a flotation bath to 48 °C for 30 minutes. Prepare the developer solution at this point. (See *Note*)
3. Flood sections, that have been laid across glass rods, with the developer solution which *must be used as soon as it is mixed*. Allow sections to develop until they are light brown or yellow. Check known control under the microscope. The spirochetes should be black and the background light brown or yellow. (See *Note*)
4. Wash quickly and thoroughly in hot tap water, approximately 56 °C.
5. Rinse in distilled water.
6. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad

RESULTS

Spirochetes, Donovan bodies	- black
Background	- pale yellow to light brown

REMARKS. It may be necessary to prolong development of sections for the demonstration of Donovan bodies. Certain hematogenous pigments, nuclei and melanin have a greater attraction for silver than do spirochetes, and it is difficult to stain the spirochetes in close proximity to these elements. By lowering the pH of the acidulated solution to 3.6 and prolonging the development, the spirochetes may be demonstrated in the areas of competition; however, the part of the section not containing competitive elements may be overstained and useless. Sections can be restained to increase the amount of development if microscopic observation of the known positive tissue shows pale spirochetes or none at all

Note Use paraffin coated forceps, particularly at step 2 and step 8.

REFERENCES. Kerr, D. A.: *Amer. J. Clin. Path. Tech. Suppl* 8:63-67, 1938. Copyright by Williams and Wilkins Co. (AFIP modification)

C. H. Bridges, and L. G. Luna studied permissible variations of this technic in the AFIP laboratories. Their report can be found in *Lab. Invest.* July-August, 1957.

5. Differentiate in 95% alcohol until blue ceases to come out into alcohol and erythrocytes and collagen are pink.
6. Dehydrate in absolute alcohol, clear in xylene, two changes each.
7. Mount with Permount or Histoclad

RESULTS

Nuclei	- blue
Basophile leucocyte, mast cell granules	- purple to violet
Cartilage	- purple
Erythrocytes, eosinophile granules	- pink
Cytoplasm	- blue to pink

REFERENCE. Mallory, F. B.: Pathological Technique, New York, Hafner Publishing Co., 1961, p. 196.

MAY-GRUNWALD GIEMSA METHOD

FIXATION. Zenker's or other well-fixed tissue.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

JENNER SOLUTION (STOCK)

Jenner stain, dry powder*	1.0 gm
Alcohol, methyl	400.0 ml

JENNER SOLUTION (WORKING)

Jenner solution (stock)	25.0 ml
Distilled water	25.0 ml

GIEMSA SOLUTION (STOCK)

(See page 119)

GIEMSA SOLUTION (WORKING)

Giemsa solution (stock)	50 drops
Distilled water	50.0 ml

Make fresh, do not re-use.

1% GLACIAL ACETIC WATER SOLUTION

(See page 94)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Remove mercuric chloride crystals with iodine and clear with sodium thiosulfate (see page 41).

*Nanonal Aniline Certified

8. Wash in running water for 10 minutes.
4. Rinse in distilled water, two changes
5. Methyl alcohol, two changes for 3 minutes each.
6. Working Jenner solution for 6 minutes.
7. Working Giemsa solution for 45 minutes
8. Handle each slide individually in this and subsequent steps. Differentiate in glacial acetic-water solution then check microscopically for well differentiated nuclei.
9. Rinse in distilled water.
10. Dehydrate quickly in 95% alcohol, absolute alcohol, and clear with xylene, two changes each.
11. Mount with Permount or Histoclad.

RESULTS

Nuclei -blue
Cytoplasm -pink to rose
Bacteria -blue

REFERENCE. Strumia, M. M. *J. Lab. Clin. Med* 21:980-984, 1935-1936.

MALLORY'S METHOD FOR HEMOFUCHSIN

FIXATION. Zenker's solution, absolute alcohol or 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

ALUM HEMATOXYLIN SOLUTION

Hematoxylin	1.0 gm
Aluminum ammonium or potassium sulfate	20.0 gm
Distilled water	400.0 ml
Thymol	1.0 gm

0.5% BASIC FUCHSIN SOULTION

Basic fuchsin	0.5 gm
Alcohol, 95%	50.0 ml
Distilled water	50.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Alum hematoxylin solution until the nuclei stand out sharply.
3. Wash thoroughly in water.
4. Basic fuchsin solution for 30 minutes.
5. Wash in water.
6. Differentiate in 95% alcohol until hemofuchsin granules stand out sharply against a gray background.
7. Dehydrate in absolute alcohol, then clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

Theory and Practice of Histological techniques

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Notes

a. The blue counterstain may be patchy if extensive caseation is present. Care should be taken not to over-counterstain as scant organisms can easily be obscured in this way.
b. Decalcification using strong acid can destroy acid fastness; formic acid is recommended.
c. Victoria blue can be substituted for carbol fuchsin and picric acid for the counterstain if colour blindness causes a recognition problem.

Cold ZN method for tubercle bacilli (Kinyoun, 1915)

Sections
Formalin-fixed, paraffin.

Staining solution

Basic fuchsin	4 g
Phenol crystals	5 g
95 per cent alcohol	20 ml
Distilled water	100 ml

Dissolve the basic fuchsin in the alcohol, and mix with the phenol and distilled water. Filter and add 1 drop of Teepol to every 30 ml of the solution.

Method

1. Take sections to water.
2. Stain in filtered carbol fuchsin solution at room temperature, 20 min.
3. Wash in tap water, and differentiate in 1 per cent acid alcohol, controlling microscopically.
4. Wash in tap water, 5–10 min.
5. Counterstain in 0.2 per cent methylene blue, 30 seconds.
6. Blot, dehydrate, clear and mount in DPX.

Results
As for standard technique

Fluorescent method for tubercle bacilli (Kuper & May, 1960)

Sections
Formalin-fixed, paraffin.

Staining solution

Auramine O	1.5 g
Rhodamine B	0.75 g
Glycerol	75 ml
Phenol crystals (liquified at 50°C)	10 ml
Distilled water	50 ml

Method

1. Take section to water (using a mixture of 1 part groundnut oil and 2 parts xylene to remove wax for *M. leprae*).
2. Pour on preheated staining solution, filtered and at 60°C, 10 min.
3. Wash in tap water.
4. Differentiate in 0.5 per cent hydrochloric acid in alcohol for *M. tuberculosis*, or 0.5 per cent aqueous hydrochloric acid for *M. leprae*.
5. Wash in tap water, 2 min.
6. Quench background fluorescence in 0.5 per cent potassium permanganate, 2 min.
7. Wash in tap water and blot dry.
8. Dehydrate (not for *M. leprae*), clear and mount in a fluorescence-free mountant.

Results
Using blue light fluorescence (below 530 nm):
Tubercle or leprosy bacilli — *golden yellow*
Background — *dark green*

Notes

a. The advantage of increased sensitivity of this technique is offset by the inconvenience of setting up the fluorescence microscope.
b. Preparations fade over time, depending on their exposure to UV light.

Wade–Fite technique for leprosy bacilli (Wade 1957, modified)

Sections
Paraffin, formalin-fixed

Solutions
As for ZN technique

Method

1. Warm the sections and dewax using a mixture of 1 part groundnut oil or clove oil and 2 parts xylene to remove wax, 10 min.
2. Repeat blotting and washing in water until section is uniformly wetted.

Gram method for bacteria in smears

Method

1. Fix the dry film by passing it three times through a flame.
2. Stain with 1 per cent crystal violet or methyl violet, 15 seconds, then pour off the excess stain.
3. Flood with Lugol's iodine, 30 seconds, then pour off excess.
4. Flood with acetone for not more than 2–5 seconds; wash with water immediately. Alternatively, decolorise with alcohol until no more stain comes away; wash with water.
5. Counterstain with dilute carbol fuchsin, 20 seconds, or neutral red (freshly filtered) 1–2 min.
6. Wash with water and blot dry.

Results

Gram-positive organisms — *blue-black*
Gram-negative organisms — *red*

Gram stain for paraffin sections (Gram, 1884)

Sections

Formalin-fixed, paraffin.

Solutions

a. Crystal violet solution:
0.5 per cent crystal violet in 25 per cent alcohol

b. Gram's and Lugol's iodine

Iodine	1 g
Potassium iodide	2 g
Distilled water	10 ml

Shake or grind until dissolved, make up to 300 ml, with distilled water, for Gram's iodine or 100 ml for Lugol's iodine

c. 1 per cent aqueous neutral red

Method

1. Take sections to water
2. Stain with filtered crystal violet solution, 2 min.
3. Rinse in tap water and drain.
4. Pour on the iodine solution b, 2 min.
5. Rinse in tap water, blot and flood with acetone, 1–2 seconds
6. Wash in tap water.
7. Counterstain in neutral red, 3 min.
8. Blot, dehydrate rapidly, clear and mount in DPX

Results

Gram-positive organisms, fibrin, some fungi, Paneth cell granules, keratohyalin and keratin — *blue*
Gram-negative organisms — *red*

Gram-Twort stain (Twort, 1924; Ollet, 1947)

Solutions

a. Crystal violet solution
As for previous method.

b. Gram's iodine
As for previous method.

c. Twort's stain

0.2 per cent neutral red in ethanol	9 ml
0.2 per cent fast green in ethanol	1 ml
Distilled water	30 ml

Mix immediately before use.

Method

1. Dewax in xylene, hydrate through graded alcohols to water.
2. Stain in crystal violet solution, 3 min.
3. Rinse in running tap water.
4. Treat with Gram's iodine, 3 min.
5. Rinse in tap water, blot dry, and complete drying in a warm place.
6. Differentiate in preheated acetic alcohol (2 per cent acetic acid in absolute alcohol) at 56°C until no more colour washes out. This may take 15–20 min; the section should be a light brown or straw colour. ← (*)
7. Rinse briefly in distilled water.
8. Stain in Twort's stain, 5 min.
9. Wash in distilled water.
10. Rinse in acetic alcohol, until no more red leaves the section, this should take only a few seconds.

11. Rinse in clean alcohol, clear in xylene and mount in DPX

Results
Gram-positive organisms — *blue-black*
Gram-negative organisms — *pink to red*
Nuclei — *red*
Red blood cells and most cytoplasmic structures — *green*
Elastic fibres — *black*

Note
Twort's stain can be used with effect as a counterstain in the basic method on p. 294, instead of neutral red. Again the green counterstain facilitates the detection of the red-staining Gram-negative organisms.

TECHNIQUES FOR MYCOBACTERIA

These organisms are difficult to demonstrate by the Gram technique because they possess a capsule containing a long-chain fatty acid, mycolic acid, which makes them hydrophobic. This fatty capsule influences the penetration and resistance to removal of stain by acid and alcohol (acid- and alcohol-fast), and is of variable robustness between the various species which make up this group. Phenolic acid and, frequently, heat are used to reduce the surface tension, increasing porosity and forcing dyes to penetrate this capsule. The speed of removal by differentiation with acid/alcohol of the primary dye is proportional to the extent of the fatty coat. The avoidance of defatting agents such as alcohol and xylene in methods for *M. leprae* are an attempt to conserve its fragile fatty capsule.

Mycobacteria are PAS-positive due to the carbohydrate present in their cell walls. This positivity is only evident when large concentrations of the organisms are present. When organisms die they lose their fatty capsule and consequently their ZN positivity. The carbohydrate can still be demonstrated by the Grocott methenamine silver reaction which may prove useful when the ZN fails, particularly if the patient is already receiving TB therapy.

A possible source of contamination may be found growing in the glutinous material lining some taps and connected rubber tubing. These organisms are acid- and alcohol-fast but are usually easily identified as contaminants by their appearance as clumps above the focal plane of the section, i.e. floaters.

Ziehl–Neelsen stain for tubercle bacilli (1882, 1883)

Fixation
Formalin or any except Carnoy's

Section
Paraffin

Reagents required

a. Carbol-fuchsin
1 g of basic fuchsin is dissolved in 10 ml of absolute alcohol, and 100 ml of 5 per cent aqueous phenol is added. Mix well. Filter before use.

b. Acidified methylene blue
0.25 per cent methylene blue in 1 per cent acetic alcohol.

Method
1. Dewax in xylene and hydrate through graded alcohols to water.
2. Flood section with freshly filtered carbol fuchsin and heat to steaming (by intermittent flaming), 15 min, OR stain in a Coplin jar at 56–60°C (oven or water bath), 30 min ← O
3. Wash well in tap water
4. Differentiate in 1 per cent acid alcohol, 10 min.
5. Wash in tap water, 5–10 min
6. Counterstain in methylene blue solution, 30 seconds.
7. Blot, and differentiate by alternate dehydration and rehydration until the background is a delicate pale blue. ← *
8. Finally dehydrate, clear and mount in DPX

Results
Tubercle bacilli, hair shafts, Russell bodies, Splendore–Hoeppli immunoglobulin around Actinomyces and some fungal organisms — *red*
Background — *pale blue*